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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Caput et al Examiner : D. Schmickel
Serial n° 07/659,408 Art Unit 1814
Filed : April 25, 1991
For : URATE OXIDASE ACTIVITY PROTEIN, RECOMBINANT GENE CODING
 THEREFOR, EXPRESSION VECTOR, MICROORGANISMS AND TRANS-
 FORMED CELLS

Honorable Commissioner of
Patent and Trademarks
Washington, D.C. 20231

ELIZABETH LARBRE DECLARATION PURSUANT TO 37. C.F.R. §.132

Elizabeth Larbre, Ph. D., declares that :

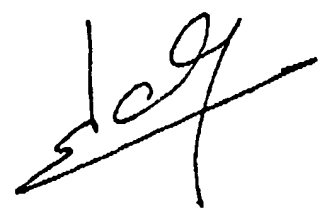
1. (see enclosed curriculum vitae)
2. I am a co-named inventor of U.S. Serial N° 07/659,408 entitled URATE OXIDASE ACTIVITY PROTEIN, RECOMBINANT GENE CODING THEREFOR, EXPRESSION VECTOR, MICROORGANISMS AND TRANSFORMED CELLS by Caput et al.
3. I have reviewed U.S. Patent 3,810,820 by Laboureur et al., U.S. Patent 4,431,739 by Riggs, Reddy et al., Proc. Natl. Acad. Sci. USA 85:9081-9085 (1988), Nielsen et al, Proc. Natl. Acad. Sci. USA 80:5198-5202 (1983), as well as the application named in paragraph 2, above. The following statements are formed upon my understanding of these documents and my personal knowledge of the field.
4. The Laboureur patent describes a process for the production of urate oxidase, an enzyme responsible for conversion of uric acid to allantoin. This production involves the purification of the enzyme from bacteria, yeasts, or other fungi. Generally, this process starts by pulverizing microbiological mass containing endocellular urate oxidase and subjecting the product to a solid-

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liquid extraction procedure. Extraction involves removal of non-urate oxidase proteins by dessication or salt complexing. Further, urate oxidase, being soluble in water but insoluble in organic solvents or concentrated aqueous solutions of inorganic salts, can be recovered by precipitation or salting out. If salts are used, they must be removed by dialysis or rapid filtration.

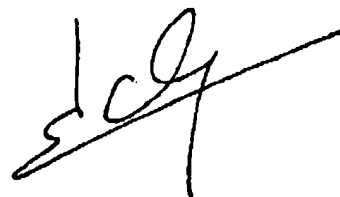
5. The extraction described in the preceding paragraph results in a urate oxidase preparation exhibiting a specific activity of approximately 8 U/mg. The lack of purity of that urate oxidase extract, and the potential advantage of having a more pure form of urate oxidase, were recognized at the time by Laboureur et al. and others. Despite this knowledge, no such product was forthcoming until the present invention.

6. After the Laboureur patent issued, affinity chromatography was developed. This approach employs a specific binding partner of the protein to be purified to hold the protein to a column, after which the protein must be eluted and recovered. Our initial attempts to further purify urate oxidase employed affinity chromatography using xanthine, a competitive inhibitor of urate oxidase, as a binding partner. Use of either 10 mM borax or carbonate buffers pH 9.0 failed to produce binding of the enzyme to the gel. 10 mM sodium bicarbonate buffer pH 8.3 did allow binding but yield was low (30-40 %) because the binding was too tight. Addition of saline to the elution buffer improved yield but this mode of purification also resulted in destabilization and inactivation of the recovered enzyme. Elution with a dissolved xanthine gradient resulted in better and more stable elution, but was hard to follow spectroscopically. The xanthine needed to be removed, and uric acid was for this purpose. Results, however, were not favorable. By this time, pseudo-affinity chromatography had arrived on the scene and further attempts to use affinity chromatography were abandoned.



7. Pseudo-affinity chromatography employs proteins-binding ligands to attach proteins to columns, similar to affinity chromatography. The ligand, however, is not a specific binding partner, thus hopefully making elution less difficult. Initial efforts using Cibacron Blue Agarose 3000 CL-L, Blue 4 Agarose, Brown 10 Agarose, Yellow 86 Agarose, and Blue Sepharose CL-6B failed to produce binding. Subsequently, only two gels have been shown to result in binding of urate oxidase - Green 19 Agarose and Red 120 Agarose. The latter gave an eluted urate oxidase of specific activity of 18 U/mg, as compared with 12 U/mg for the former. (In those preliminary experiments chromatography was performed with a with 10 mM sodium bicarbonate pH 8.3 buffer equilibrated column and an eluting solution consisting of 10 mM sodium bicarbonate pH 8.3 buffer supplemented with 1 M sodium chloride solution). Still further experimentation was needed to achieve the specific activity recited in the claims of the Caput et al. application using Red 120 Agarose. A primary sulfate cut, followed by diafiltration, was found to be required prior to affinity chromatography. Finally, following chromatography, ultragel purification to remove the major part of a contaminating dimer band of 70-71 kDa was performed. Only after these further refinements were the high specific activity urate oxidase products achieved. The final three step process is described in Example 4 of the Caput et al. application at page 15, line 4, to page 17, line 16. (In the latter process 10 mM sodium bicarbonate buffer was abandoned in favor of a 20 mM glycine/NaOH pH 8.3 buffer, which ensures stability of the purified protein).

8. In view of the preceding discussion, I believe that no one in the field could have held a reasonable expectation of successfully purifying urate oxidase based on the Laboureur patent, alone or in conjunction with Reddy et al. and Riggs or Nielsen et al., until the invention of Caput et al.



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9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true ; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date : 23/09/92

By : 
Elizabeth Larbre

Elisabeth LARBRE

Date of Birth : 11 July 1958 (Paris, France)
Address : 7, rue du rempart St. Roch, 84000 Avignon, France
Telephone : 00.85.75.66

Profession : Research and Development Engineer
Doctoral Diploma in Biotechnology

Languages : Fluent English and Italian, Knowledge of German

Hobbies : Violin ; Drawing (pastels) ; walking ; cross-country, skiing

EDUCATION

1976 Baccalaureat 'D' (Science)

1976 à 1978 University of Paris (VII)
First year studies in medicine (9 credits)

1979 D.E.U.G. (equivalent to Associate of Sciences)

1980 Degree in Biochemistry

1981 Masters degree in Biochemistry

1982 Certificate of Detailed Studies (A.E.S.A.) in Vegetable Physiology and Biology. Mark : Assez Bien (B)

1983 Diploma of Detailed Studies (D.E.A.) in Vegetable Physiology and Biology. Subject : Study, purification and characterisation of RNases of Broad Bean roots, carried out in the laboratory of Mr Robert Eenault.
Experiments carried out in Immunology at the Institute Pasteur (Mr Fellous's department)
Mark : Bien (B+)

1984 - 1987 Doctoral Diploma - French Petroleum Institute (I.F.P.) in the department run by Mr Vandecasteele and Mr Pourquié
Subject : the study of cellulase excretions of the Trichoderma reesei ; characteristics of the cellulolytic complex (strain CL 847) in relation to its industrial application.

Thesis under the direction of Mr Jacques Pourquié (I.F.P.)
President of examining jury : Mr Robert Esnault (University of
Paris VII and CNRS Phytotron, Gif-sur-Yvette)
Complimentary study course in Immunology at the Jacques Mo-
nod Institute (C.N.R.S. Paris VII)

- 1989 8 - 12 September, I.E.A. Meeting, Lund :
Presentation : 'Production and characterisation of cellulolytic en-
zymes from Trichoderma reesei grown on various carbon sour-
ces' by M. Warzywoda, E. Larbre and J. Pourquié
- 1990 April 4 1990 Doctoral Viva Voce : Doctorate awarded Highly
Commended with Distinction.

SHORT COURSES AND IN-SERVICE TRAINING

Feb. - March 1984 French Petroleum Institute, under the supervision of Mr Jean-
Paul Vendecasteele and Mr Jacques Pourquié : Course compli-
mentary to doctoral studies. Subject : study and Purification of
the cellulolytic system of the Trichoderma reesei mould (strain
CL 847) using Fast Protein Liquid Chromatography.

April 1984 - July 1987

Institute Jacques Monod, Paris VII : Immunology.

1988 Centre for Research into Industrial Growth (C.R.E.C.I.) :
One week course in Business Communication

1989 One week in-Service training course : Knowledge and Use of
Proper Fabrication Procedures

I.C.P.I. Lyon, one week course in Basic II

• Programming using Fortran (2 credits, Paris VII)

Word Processing

Framework (Amstrad PC)
 Mac Write (Mac II)
 Mac Draw (Mac II)
 Superpaint (Mac II)
 use of result analysis Software
 Microsoft Excel (Mac II)
 Stat View (Mac II)

Septembre 1989 (Applied Biosystem Co., Marseille) : Capillary Electrophoresis and Preparative Electrophoresis.

October 1989 (Germany) one week course : Protein Purification : Process Development, Optimization and Scaling Up.
 Use of Pharmacia Biopilot

November 1990 (Waters-Millipore, Paris). One week course : High Performance Liquid Chromatography.

1990 100 hours English (3 hours per week) - In-house training

April 1991 I.C.P.I., Lyon. One week course : Fermentors and Fermentation

May 1991 4 x 4 hours in-house course : Proper Fabrication Procedures.
 A detailed study.

SYMPOSIA AND CONFERENCES

1980 World Biotechnologies. Paris XVII. Joel de Rosnay

1984 - 1987 Annual meetings of the F.P.L.C. Pharmacia Club at Bois d'Arcy.
 Conférences at I.F.P. on the use of biomass, world energy, bio-substitute carburants...

July 1988 8th Microbiology Conference. Paris Conférence Centre

October 1989 International Symposium (SITEF), Toulouse
 Purification and analysis of biomolecules.

March 1990 UCLA Symposia on Molecular and Cellular Biology, Lake Tahoe, Calif. USA

September 1990 18-19/09 : Seminar : Moduline II Workshop, AMICON
 20 - 23/09 : International congress on peptide inhibitors of pro-

teolytic enzymes

WORK PROFILE

January 1988 to August 1991

Assistant Department Chief, Biotechnology Research and Development. Sanofi-Chimie Pharmaceutical Production

In charge of the Biochemical Purification Service (Creation, set-up and direction)

Training of a team of higher technicians for :

- improvement and total overhaul of fabrication processes (especially from recombinant proteins).
- creation and research of processes for the fabrication of tertiary products (especially those destined for Immuno-Diagnosis)
- development and perfection of analytical methods (biochemistry and immunology)
- fabrication assistance (dependability of processes and methods).

Results confirmed by the development of a patent :

Purification of a protein through the activity of urate-oxidase, relevant recombinant gene, vector of activity, micro-organisms and transformed cells. (European Patent registered 13.07.1990)

Laboratory supervision of engineering students from University of Marseille, St. Charles : MA Science and Technology of Industrial and Applied Microbiology - end of year course. Member of examining jury.

In charge of Analytical Development for the Production Plant.

In charge of analysis (development and control) of Biochemical Development : Laboratory and Pilot Studies.

SINCE MAY 1992 :

Expert in Biochemistry for the Department of Biotechnology Research and Development :

- advise in Biochemical Purification and Analytical Development
- actually, in charge of writing the chemical part of a report about an important Recombinant Protein, for the french Health Office

TECHNICAL SPECIALISATION :

**Protéin Extraction and Purification
Analysis/Immunology/enzymology**

**8 years experience of Research and Development methods and techniques
(Laboratory and Pilot Studies)**

PROTEIN EXTRACTION

Ultra-, dia-, and microfiltration, fractional and total precipitation systems (PEG, ammonium sulfate), biphasic systems, liquid/liquid extraction (PEG/maltodextrines or dextrine PEG/salt)

PROTEIN PURIFICATION AND BIOCHEMICAL ANALYSIS

Low and medium pressure liquid chromatography (F.L.P.C. and Bioplot using F.P.L.C. Manager Software) and high pressure liquid chromatography (H.P.L.C.)

Gel filtration, ion exchange, chromatofocusing, inverse phase analysis of metals and ions on Dionex ; (Diode bar detection, conductimetry, refractometry, saccharification operations, post-column derivation etc...)

Affinity and pseudo-affinity chromatography (Dye-ligand Chelating-Gel (I.M.A.C.), Activated Magnetic Gel) or of Immuno-affinity.

Electrophoreses (mono or bidimensional). Phastsystem Electrophoresis (Pharmacia) native or denatured conditions, electrofocusing, titration curves...

Use of two types of coloration : silver nitrate or Coomassie blue.

GENERAL AND ANALYTICAL IMMUNOLOGY

Immunisation and negativity control in relation to immunogens

Study of coupling conditions of subject proteins, using solid supports (pH, ionic Strength...)

Ouchterlony, double immunodiffusion, immuno-electrophoresis, Immuno-transfer, on nitrocellulose or nylon using antibodies coupled by peroxidase or by alkaline phosphatase.

Definition of E.L.I.S.A. doses, titration and specificity checking of antibodies.

Titration of antibodies by R.I.A. (Iode marking)

Purification of polyclonal antibodies (precipitation by ammonium sulphate, dialysis and chromatography by weak anionic exchange (D.E.A.E. Sephadex A-50), strong anionic exchange (Q Sepharose Fast Flow) or A Sepharose Protein (Pharmacia), Perflex 35S Protein A (Dupont de Nemours)...

Advanced and specific antibody purification by enzyme

immobilisation with chromatographic support.

Immuno-affinity (coupling of antibodies by matrices such as ultrogel Aca 22 preactivated to glutaraldehyde (IBF), CNBr-Activated Sepharose, Pharmacia, etc...)

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